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(54) Title: USE OF rAFP TO INHIBIT OR PREVENT APOPTOSIS

(57) Abstract: A method of inhibiting apoptosis in a cell by administering to the cell an apoptosis inhibiting amount of recombinant human alpha-feta protein or an apoptosis-inhibiting fragment thereof.

## USE OF rAFP TO INHIBIT OR PREVENT APOPTOSIS

### BACKGROUND OF THE INVENTION

5           The invention related to methods of inhibiting apoptosis.

          There are two general ways in which cells die. The most easily recognized way is by necrosis, which is usually caused by an injury that is severe enough to disrupt cellular homeostasis. Typically, the cell's osmotic pressure is disturbed and, consequently, the cell swells and then ruptures.

10          When the cellular contents are spilled into the surrounding tissue space, an inflammatory response often ensues.

          The second general way by which cells die is referred to as apoptosis, or programmed cell death. Apoptosis often occurs so rapidly that it is difficult to detect. This may help to explain why the involvement of  
15          apoptosis in a wide spectrum of biological processes has only recently been recognized.

          The apoptosis pathway has been highly conserved throughout evolution, and plays a critical role in embryonic development, viral pathogenesis, cancer, autoimmune disorders, and neurodegenerative disease.  
20          For example, inappropriate apoptosis may cause or contribute to AIDS, Alzheimer's Disease, Parkinson's Disease, Amyotrophic Lateral Sclerosis (ALS), retinitis pigmentosa and other diseases of the retina, myelodysplastic syndrome (e.g. aplastic anemia), toxin-induced liver disease, including alcoholism, and ischemic injury (e.g. myocardial infarction, stroke, and  
25          reperfusion injury). Conversely, the failure of an apoptosis response has been implicated in the development of cancer, particularly follicular lymphoma, p53-mediated carcinomas, and hormone-dependent tumors, in autoimmune

disorders, such as lupus erythematosus and multiple sclerosis, and in viral infections, including those associated with herpes virus, poxvirus, and adenovirus.

In patients infected with HIV-1, mature CD4<sup>+</sup> T lymphocytes respond to stimulation from mitogens or super-antigens by undergoing apoptosis. However, the great majority of these cells are not infected with the virus. Thus, inappropriate antigen-induced apoptosis could be responsible for the destruction of this vital part of the immune system in early stages of HIV infection.

#### SUMMARY OF THE INVENTION

In general, the invention features the inhibition of apoptosis in a cell, e.g., a cell in a mammal such as a human patient, by contacting the cell with recombinant alpha-fetoprotein ("rHuAFP") or an effective fragment thereof, or with nucleic acid encoding rHuAFP. The invention, in inhibiting apoptosis, can provide therapy for diseases in which inappropriate apoptosis is a feature, including AIDS or HIV infection, neurodegenerative diseases such as ALS, a myelodysplastic syndrome, or an ischemic injury such as occurs in stroke, myocardial infarction, reperfusion injury, or a toxin-induced liver disease. Other features and advantages of the invention will be apparent from the detailed description of the invention, the drawings, and the claims.

#### BRIEF SUMMARY OF THE DRAWINGS

Fig. 1 is the nucleotide sequence (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) of the cDNA encoding human alpha-fetoprotein, and the amino acid sequences (SEQ ID NOS: 3-8) of rHuAFP fragments.

Fig. 2 is the SDS-PAGE analysis of rHuAFP Fragment I (SEQ ID NO: 8) (Lane A, MW marker; Lane B, native human alpha-fetoprotein (AFP); Lane C, unpurified rAFP; Lane D, rAFP Fragment I, and Lane E, AFP (amino acids 1-590 of Fig. 1, SEQ ID NO: 2).

5

## DETAILED DESCRIPTION OF THE INVENTION

### Production of Recombinant Human Alpha-fetoprotein

Recombinant AFP can be produced in any standard recombinant protein production system, including prokaryotic cells such as E. coli, and eukaryotic systems such as yeast, mammalian (e.g., CHO cells) and insect cells.

10 Prokaryotic production of rHuAFP is described in Murgita U.S. Patent No. 5,384,250, hereby incorporated by reference.

The methods of the invention can also employ biologically active fragments of rHuAFP. A biologically active fragment of rHuAFP is one that possesses at least one of the following activities: (a) directs a specific  
15 interaction with a target cell, e.g., binds to a cell expressing a receptor that is recognized by rHuAFP (e.g., the membrane of a cancer cell such as MCF-7); or (b) halts, reduces, or inhibits apoptosis (e.g., binds to a cell surface receptor and imparts an anti-apoptosis signal). The ability of rHuAFP fragments to bind to a receptor which is recognized by rHuAFP can be tested using any  
20 standard binding assay known in the art.

In general, fragments of rHuAFP are produced according to the techniques of polypeptide expression and purification described in U.S. Patent No. 5,384,250. DNA sequences encoding fragments of rHuAFP can be generated by standard techniques and cloned into expression vectors for  
25 expression in recombinant cells. Expressed fragments can be isolated by various chromatographic and/or immunological methods known in the art.

Lysis and fractionation of rHuAFP-containing cells prior to affinity chromatography may be performed by standard methods. Once isolated, the recombinant protein can, if desired, be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher, Laboratory Techniques  
5 In Biochemistry and Molecular Biology, Work and Burdon, eds., Elsevier, 1980).

Recombinant HuAFP fragments can be assayed by immunological procedures, such as Western blot, immunoprecipitation analysis of recombinant cell extracts, or immunofluorescence (using, e.g., the methods  
10 described in Ausubel et al., *Current Protocols In Molecular Biology*, Greene Publishing Associates and Wiley Interscience (John Wiley & Sons), New York, 1994).

Useful rHuAFP fragments preferably have at least 20 contiguous amino acids, preferably at least 50 contiguous amino acids, more preferably at  
15 least 100 contiguous amino acids, and most preferably at least 200 to 400 or more contiguous amino acids in length.

Recombinant HuAFP fragments of interest include, but are not limited to, Domain I (amino acids 1 (Thr) - 197 (Ser), see Fig. 1, SEQ ID NO: 3), Domain II (amino acids 198(Ser) - 389 (Ser), see Fig. 1, SEQ ID NO: 4),  
20 Domain III (amino acids 390 (Gln) - 590 (Val), see Fig. 1, SEQ ID NO: 5), Domain I+II (amino acids 1 (Thr) - 389 (Ser), see Fig. 1, SEQ ID NO: 6), Domain II+III (amino acids 198 (Ser) - 590 (Val), see Fig. 1, SEQ ID NO: 7), and rHuAFP Fragment I (amino acids 266 (Met) - 590 (Val), see Fig. 1, SEQ ID NO: 8).

25 By "inhibiting apoptosis" is meant a decrease in the number of cells which undergo apoptosis relative to an untreated control. Preferably, the decrease is at least 25%, more preferably the decrease is 50%, and most

preferably the decrease is at least one-fold.

### Apoptosis Assays

- Apoptosis assays are described in the following references. Assays for apoptosis in lymphocytes are disclosed by, for example: Li et al.,
- 5 "Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein", Science 268:429-431, 1995; Gibellini et al., "Tat-expressing Jurkat cells show an increased resistance to different apoptosis stimuli, including acute human immunodeficiency virus-type 1 (HIV-1) infection", Br. J. Haematol. 89:24-33, 1995; Martin et al., "HIV-1 infection of human CD4<sup>+</sup> T cells *in vitro*.
- 10 Differential induction of apoptosis in these cells." J. Immunol. 152:330-42, 1994; Terai et al., "Apoptosis as a mechanism of cell death in cultured T lymphoblasts acutely infected with HIV-1", J. Clin Invest. 87:1710-5, 1991; Dhein et al., "Autocrine T-cell suicide mediated by APO-1/(Fas/CD95) 11, Nature 373:438-441, 1995; Katsikis et al., "Fas antigen stimulation induces
- 15 marked apoptosis of T lymphocytes in human immunodeficiency virus-infected individuals", J. Exp. Med. 181:2029-2036, 1995; Estendorp et al., "Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120", Nature 375:497, 1995; DeRossi et al., Virology 198:234-44, 1994.

- Assays for apoptosis in fibroblasts are disclosed by, for example:
- 20 Vossbeck et al., "Direct transforming activity of TGF-beta on rat fibroblasts," Int. J. Cancer 61:92-97, 1995; Goruppi et al., "Dissection of c-myc domains involved in S phase induction of NIH3T3 fibroblasts," Oncogene 9:1537-44, 1994; Fernandez et al., "Differential sensitivity of normal and Ha-ras transformed C3H mouse embryo fibroblasts to tumor necrosis factor: induction
- 25 of bcl-2, c-myc, and manganese superoxide dismutase in resistant cells," Oncogene 9:2009-17, 1994; Harrington et al., "c-Myc-induced apoptosis in

fibroblasts is inhibited by specific cytokines," EMBO J., 13:3286-3295, 1994;  
Itoh et al., "A novel protein domain required for apoptosis. Mutational  
analysis of human Fas antigen," J. Biol. Chem. 268:10932-7, 1993.

Assays for apoptosis in neuronal cells are disclosed by, for example:

- 5 Melino et al., "Tissue transglutaminase and apoptosis: sense and antisense  
transfection studies with human neuroblastoma cells," Mol. Cell Biol.  
14:6584-6596, 1994; Rosenbaum et al., "evidence for hypoxia-induced,  
programmed cell death of cultured neurons," Ann. Neurol. 36:864-870, 1994;  
Sato et al., "Neuronal differentiation of PC12 cells as a result of prevention of  
10 cell death by bcl-2," J. Neurobiol. 25:1227-1234, 1994; Ferrari et al., "N-  
acetylcysteine D- and L-stereoisomers prevents apoptosis death of neuronal  
cells," J. Neurosci. 15:2857-2866, 1995; Talley et al., "Tumor necrosis  
factor alpha-induced apoptosis in human neuronal cells: protection by the  
antioxidant N-acetylcysteine and the genes bcl-2 and crmA," Mol. Cell Biol.  
15 1585:2359-2366, 1995; Talley et al., "Tumor Necrosis Factor Alpha-Induced  
Apoptosis in Human Neuronal Cells: Protection by the Antioxidant  
N-Acetylcysteine and the Genes bcl-2 and crmA," Mol. Cell. Biol. 15:2359-  
2366, 1995; and Walkinshaw et al., "Induction of apoptosis in  
catecholaminergic PC12 cells by L-DOPA. Implication for the treatment of  
20 Parkinson's disease," J. Clin. Invest. 95:2458-2464, 1995.

Assays for apoptosis in insect cells are disclosed by, for example:

- Clem et al., "Prevention of apoptosis by a baculovirus gene during infection of  
insect cells," Science 254:1388-90, 1991; Crook et al., "An apoptosis-  
inhibiting baculovirus gene with a zinc finger-like motif," J. Virol. 67:2168-  
25 74, 1993; Rabizadeh et al., "Expression of the baculovirus p35 gene inhibits  
mammalian neural cell death," J. Neurochem. 61:2318-21, 1993; Birnbaum et  
al., "an apoptosis inhibiting gene from a nuclear polyhedrosis virus encoding a

polypeptide with Cys/His sequence motifs," J. Virol. 68:2521-8, 1994; and Clem'et al., "Control of programmed cell death by the baculovirus genes p35 and IAP," Mol. Cell. Biol. 14:5212-5222, 1994.

### Gene Therapy

- 5           rHuAFP-encoding genes can be used according to the invention in anti-apoptosis gene therapy. In particular, a functional rHuAFP gene may be used to sustain neuronal cells that undergo apoptosis in the course of a neurodegenerative disease; lymphocytes (i.e., T cells and B cells); or cells that have been injured by ischemia.
- 10           Retroviral vectors, adenoviral vectors, adeno-associated viral vectors, or other viral vectors with the appropriate tropism for cells likely to be involved in apoptosis (for example, epithelial cells) may be used as a gene transfer delivery system for a therapeutic rHuAFP gene construct. Numerous vectors useful for this purpose are known (Miller, Human Gene Therapy 15-15, 1990; Friedman, Science 244:1275-1281, 1989; Eglitis and Anderson, BioTechniques 6:608-614, 1988; Tolstoshev and Anderson, Current Opinion in Biotechnology 1:55-61, 1990; Sharp, The Lancet 337:1277-1278, 1991; Cornetta et al., Nucleic Acid Research and Molecular Biology 36:311-322, 1987; Anderson, Science 226:401-409, 1984; Moen, Blood Cells 17:407-416, 20 1991; Miller et al., Biotechniques 7:980-990, 1989; Le Gal La Salle et al., Science 259:988-990, 1993; and Johnson, Chest 107:77S-83S, 1995).
- 25           Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg et al., N. Engl. J. Med 323:370, 1990; Anderson et al., U.S. Patent NO. 5,399,346). Non-viral approaches may also be employed for the introduction of therapeutic DNA into cells otherwise predicted to undergo apoptosis. For example rHuAFP may be introduced into a neuron or a

T cell by lipofection (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413, 1987; Ono et al., Neurosci. Lett. 117:259, 1990; Brigham et al., Am. J. Med. Sci. 298:278, 1989; Staubinger et al., Meth. Enz. 101:512, 1983),  
asialorosonucoid-polylysine conjugation (Wu et al., J. Biol. Chem. 263:14621,  
5 1988; Wu et al., J. Biol. Chem. 264:16985, 1989); or, less preferably,  
microinjection under surgical conditions (Wolff et al., Science 247:1465,  
1990).

For any of the methods described above, the therapeutic rHuAFP  
DNA construct is preferably applied to the site of the predicted apoptosis event  
10 (for example, by injection), or to tissue in the vicinity of the predicted  
apoptosis event, or to a blood vessel supplying the cells predicted to undergo  
apoptosis.

rHuAFP expression can be directed from any suitable promoter (e.g.,  
the human cytomegalovirus (CMV), simian virus 40 (SV40), or  
15 metallothionein promoters), and regulated by any appropriate mammalian  
regulatory element. For example, if desired, enhancers that preferentially  
direct gene expression in neural cells, T cells, or B cells may be used to direct  
rHuAFP expression. Alternatively, if an rHuAFP genomic clone is used in a  
therapeutic construct, regulation may be mediated by the cognate regulatory  
20 sequences or, if desired, by regulatory sequences derived from a heterologous  
source, including any of the promoters or regulatory elements described above.

Alternatively, rHuAFP gene therapy is accomplished by  
direct administration of the rHuAFP mRNA or antisense rHuAFP mRNA to a  
cell that is expected to undergo apoptosis. The mRNA may be produced and  
25 isolated by any standard technique, but is most readily produced by *in vitro*  
transcription using an rHuAFP cDNA under the control of a high efficiency  
promoter (e.g., the T7 promoter). Administration of rHuAFP mRNA to cells

can be carried out by any of the methods for direct nucleic acid administration described below.

Ideally, the production of rAFP protein by any gene therapy approach will result in cellular levels of rAFP that are at least equivalent to the normal, cellular level of rHuAFP in an unaffected cell. Treatment by any rHuAFP-mediated gene therapy approach may be combined with more traditional therapies.

#### Administration of rAFP Polypeptides

Another therapeutic approach of the invention involves administration of recombinant rHuAFP, either directly to the site of a predicted apoptosis event (for example, by injection) or systemically (for example, by any conventional recombinant protein administration technique). The dosage of rHuAFP depends on a number of factors, including the size and health of the individual patient, but, generally, between 0.1 mg and 100 mg are administered per day to an adult in a pharmaceutically-acceptable formulation. Administration may begin before or after the patient is symptomatic. Any appropriate route of administration may be employed, for example, administration may be parenteral, intravenous, intraarterial, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or oral. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art of making formulations are found, for example, in *Remington's Pharmaceutical Sciences*, (18<sup>th</sup> edition), ed. A.

Gennaro, 1990, Mack Publishing Company, Easton, PA. Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated naphthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of rHuAFP.

Treatment with an rHuAFP protein or gene may be combined with more traditional therapies for the disease such as surgery, steroid therapy, or chemotherapy for autoimmune disease; antiviral therapy for AIDS; and tissue plasminogen activator (TPA) for ischemic injury.

#### Other Embodiments

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth, and follows in the scope of the appended claims.

What is claimed is:

1. A method of inhibiting apoptosis in a cell, said method comprising administering to said cell an apoptosis inhibiting amount of rHuAFP or an apoptosis-inhibiting fragment thereof.
2. The method of claim 1, wherein said cell is in a mammal.
- 5 3. The method of claim 2, wherein said mammal is human.
4. The method of claim 3, wherein said human is infected with HIV, or has a neurodegenerative disease, a myelodysplastic syndrome, or an ischemic injury.
5. The method of claim 4, wherein said ischemic injury is  
10 caused by a myocardial infarction, a stroke, a reperfusion injury, or a toxin-induced liver disease.
6. A method of inhibiting apoptosis in a cell, said method comprising transfecting said cell with nucleic acid encoding rHuAFP or an apoptosis-inhibiting fragment thereof.
- 15 7. The method of claim 6, wherein said cell is in a human patient.
8. The method of claim 7, wherein said human patient is infected with HIV, or has a neurodegenerative disease, a myelodysplastic syndrome, or an ischemic injury.

AT (2)  
 -1  
 met lys trp val glu ser ile phe leu ile phe leu asn phe thr glu ser arg  
 -10  
 ATG AAG TGG GTG GAA TCA ATT TTT TTA ATT TTC CTA AAT TTT ACT GAA TCC AGA (101)  
 ATTTGCTTCCACCACTGCCAATAACAAATAACTAGCAACC  
 -19  
 met lys trp val glu ser ile phe leu ile phe leu asn phe thr glu ser arg  
 10  
 thr leu his arg asn glu tyr gly ile ala ser ile leu asp ser tyr gln cys thr ala glu ile ser leu ala asp leu ala thr ile  
 ACA CTG CAT AGA AAT GAA TAT GGA ATA GCT TCC ATA TTG GAT TCT TAC CAA TGT ACT GCA GAG ATA AGT TTA GCT GAC CTG ACC ATA (191)  
 30  
 phe phe ala gln phe val gln glu ala thr tyr lys glu val ser lys met val lys asp ala leu thr ala ile glu lys pro thr gly  
 40  
 TTT TTT GCC CAG TCT TCA GGG TGT TTA GAA AAC CAG CTA CCT GCC TTT CTG GAA GAA CTT TGC CAT GAG AAA GAA CCC ACT GGA (281)  
 50  
 60  
 asp glu gln ser ser gly cys leu glu asn gln leu pro ala phe leu glu glu leu cys his glu lys glu ile leu glu lys tyr gly  
 70  
 GAT GAA GAG TCT TCA GGG TGT TTA GAA AAC CAG CTA CCT GCC TTT CTG GAA GAA CTT TGC CAT GAG AAA GAA CCC ACT GGA (371)  
 80  
 90  
 his ser asp cys cys ser gln ser glu glu gly arg his asn cys phe leu ala his lys lys pro thr pro ala ser ile pro leu phe  
 100  
 CAT TCA GAC TGC TGC AGC CAA AGT GAA GAG GAG GAG CAC AAA AAG CCC ACT CCA GCA TCG ATC CCA CTT TTC (461)  
 110  
 120  
 gln val pro glu pro val thr ser cys glu ala tyr glu glu asp arg glu thr phe met asn lys phe ile tyr glu ile ala arg arg  
 130  
 CAA GTT CCA GAA CCT GTC ACA AGC TGT GAA GCA TAT GAA GAA GAC AGG GAG ACA TTC ATG AAC AAA TTC ATT TAT GAG ATA GCA AGA AGG (551)  
 140  
 150  
 his pro phe leu tyr ala pro thr ile leu leu trp ala ala arg tyr asp lys ile pro ser cys cys lys ala glu asn ala val  
 160  
 CAT CCC TTC CTG TAT GCA CCT ACA ATT CTT TGG GCT GCT CGC TAT GAC AAA ATA ATT CCA TCT TGC TGC AAA GCT GAA AAT GCA GTT (641)  
 170  
 180  
 glu cys phe gln thr lys ala ala thr val thr lys glu leu arg glu ser ser leu leu asn gln his ala cys ala val met lys asn  
 190  
 GAA TGC TTC CAA ACA AAG GCA GCA ACA GTT ACA AAA GAA TTA AGA GAA AGC AGC TTG TTA AAT CAA CAT GCA TGT GCA GTA ATG AAA AAT (731)  
 200  
 210

Fig. 1

211 phe gly thr arg thr phe gln ala ile thr val thr lys leu ser gln lys phe thr lys val asn phe thr glu ile gln lys leu val 240  
 TTT GGG ACC CGA ACT TTC CAA GCC ATA ACT GTT ACT AAA CTG AGT CAG AAG TTT ACC AAA GTT AAT TTT ACT GAA ATC CAG AAA CTA GTC (821)  
 241 leu asp val ala his val his glu his cys cys arg gly asp val leu asp cys leu gln asp gly lys ile met ser tyr ise cys 270  
 CTG GAT GTG GCC CAT GTA CAT GAG CAC TGT TGC AGA GGA GAT GTG CTG GAT TGT CTG CAG GAT GGG GAA AAA ATC ATG TCC TAC ATA TGT (911)  
 271 ser gln gln asp thr leu ser asn lys ile thr glu cys cys lys leu thr thr leu glu arg gly gln cys ile ile his ala glu asn 300  
 TCT CAA CAA GAC ACT CTG TCA AAC AAA ATA ACA GAA TGC TGC AAA CTG ACC CTG GAA CGT GGT CAA TGT ATA ATT CAT GCA GAA AAT (1001)  
 301 asp glu lys pro glu gly leu ser pro asn leu ser arg phe leu gly asp arg asp phe asn gln phe ser ser gly glu lys asn ile 330  
 GAT GAA AAA CCT GAA GGT CTA TCT CCA AAT CTA AAC AGG TTT TTA GGA GAT AGA GAT TTT AAC CAA TTT TCT TCA GGG GAA AAA AAT ATC (1091)  
 331 phe leu ala ser phe val his glu tyr ser arg arg his pro gln leu ala val ser val ile leu arg val ala lys gly tyr gln glu 360  
 TTC TTG GCA AGT TTT GTT CAT GAA TAT TCA AGA AGA CAT CCT CAG CTT GCT GTC TCA GTA ATT CTA AGA GTT GCT AAA GGA TAC CAG GAG (1181)  
 361 leu glu lys cys phe gln thr glu asn pro leu glu cys gln asp lys gly glu glu leu gln lys tyr ile gln glu ser gln 390  
 TTA TTG GAG AAG TGT TTC CAG ACT GAA AAC CCT CTT GAA TGC CAA GAT AAA GGA GAA GAA TTA CAG AAA TAC ATC CAG GAG AGC CAA (1271)  
 391 ala leu ala lys arg ser cys gly leu phe gln lys leu gly glu tyr tyr leu gln asn ala phe leu val ala tyr thr lys lys ala 420  
 GCA TTG GCA AAG CGA AGC TGC GGC CTC TTC CAG AAA CTA GGA GAA TAT TAC TTA CAA AAT GCG TTT CTC GTT TAC ACA AAG AAA GCC (1361)  
 421 pro gln leu thr ser ser glu leu met ala ile thr arg lys met ala ala thr ala ala thr cys cys gln leu ser glu asp lys leu 450  
 CCC CAG CTG ACC TCG TCG GAG CTG ATG GCC ATC ACC AGA AAA ATG GCA GCC ACA GCA GCC ACT TGT TGT GGC CAA CTC AGT GAG GAC AAA CTA (1451)

Fig. 1 (CONTINUED)

3/4

451 leu ala cys gly glu gly ala ala asp ile ile ile gly his leu cys ile arg his glu met thr pro val asn pro gly val gly gln 480  
 TTG GCC TGT GGC GAG GGA GCG GCT GAC ATT ATT ATC GGA CAC TTA TGT ATC AGA CAT GAA ATG ACT CCA GTA AAC CCT GGT GTT GGC CAG (1541)  
 481 cys cys thr ser ser tyr ala asn arg arg pro cys phe ser ser leu val val asp glu thr tyr val pro pro ala phe ser asp asp 510  
 TGC TGC ACT TCT TCA TAT GCC AAC AGG AGG AGG CCA TGC TTC AGC AGC TTT GTC GAT GAA ACA TAT GTC CCT CCT GCA TTC TCT GAT GAC (1631)  
 511 lys phe ile phe his lys asp leu cys gln ala gln gly val ala leu gln thr met lys gln glu phe leu ile asn leu val lys gln 540  
 AAG TTC ATT TTC CAT AAG GAT CTG TGC CAA GCT CAG GGT GTA GCG CTG CAA ACG ATG AAG CAA GAG TTT CTC ATT AAC CTT GTG AAG CAA (1721)  
 541 lys pro gln ile thr glu glu gln leu glu ala val ile ala asp phe ser gly leu leu glu lys cys gln gly gln glu gln glu 570  
 AAG CCA CAA ATA ACA GAG GAA CAA CTT GAG GCT GTC ATT GCA GAT TTC TCA GGC CTG TTG GAG AAA TGC TGC CAA GGC CAG GAA CAG GAA (1811)  
 571 val cys phe ala glu glu gly gln lys leu ile ser lys thr arg ala ala leu gly val ter 590  
 GTC TGC TTT GCT GAA GAG GGA CAA AAA CTG ATT TCA AAA ACT CGT GCT GCT TTG GGA GTT TAA ATTACTTCAGGGGAGAGAGACAAACAGGAGTCT (1908)  
 TTCATTGGGTGAACTTTCTCTTTAATTTAACTGATTAACTTTTGTGATTAATGAATGATAAGACTTTTATGTGAGATTCTTATCAGAAATAAATAATCTCCAAA (2027)

Fig. 1 (CONTINUED)

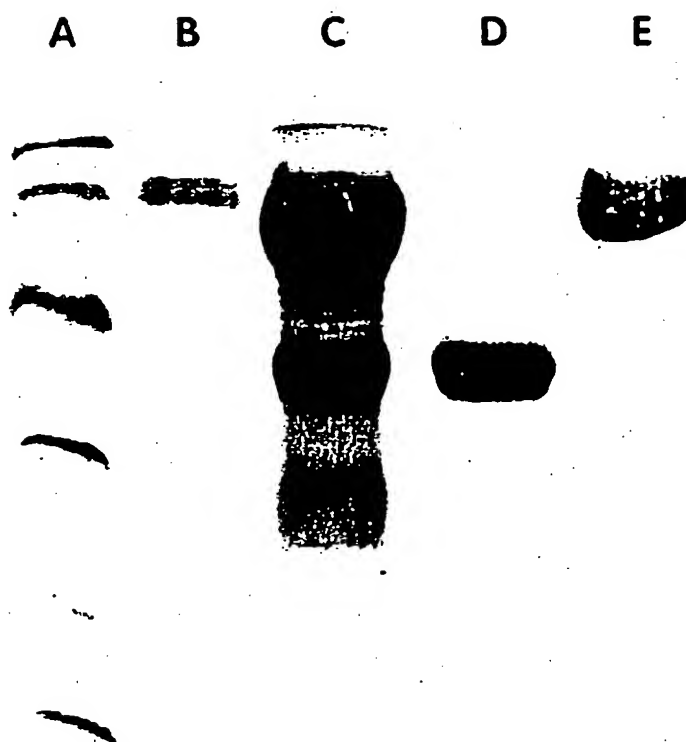


Fig. 2

## SEQUENCE LISTING

<110> Atlantic Biopharmaceuticals, Inc.  
Murgita, Robert A.  
Mulroy, Robert  
Lindsay, Stace

<120> USE OF rAFP TO INHIBIT OR PREVENT  
APOPTOSIS

<130> 06727/010W02

<150> US 60/152,166

<151> 1999-09-02

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PCT/US00/24129

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&lt;212&gt; PRT

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&lt;213&gt; Homo sapiens

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# INTERNATIONAL SEARCH REPORT

I. national application No.  
PCT/US00/24129

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 31/7088, 38/38; C12N 5/00, 15/85

US CL : 435/375, 455: 514/2, 44

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/375, 455: 514/2, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SEMENKOVA et al. Induction of apoptosis in human hepatoma cells by alpha-fetoprotein. Tumor Biol. 1997, Vol. 18, No. 5, pages 261-273, especially page 264.	1
Y	LADERROUTE et al. Role of AFP and 67 kD AFPr isoforms in the abrogation of apoptosis. Tumor Biol. 1996, Vol. 17, No. Suppl. 1, page 10, see entire document.	1
Y	DUDICH et al. The inhibition of TNF-induced apoptosis by human alpha-fetoprotein. Anticancer Res. 17-22 October 1995, Vol. 15, No. 5A, pages 1728-1729, see entire document.	1

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* "A"	Document defining the general state of the art which is not considered to be of particular relevance	* "T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* "E"	earlier document published on or after the international filing date	* "X"	document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* "L"	document which may throw doubts on priority claims or of which is cited to establish the publication date of another citation or other special reason (as specified)	* "Y"	document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* "O"	document referring to an oral disclosure, use, exhibition or other means	* "Z"	document member of the same patent family
* "P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

15 DECEMBER 2000

Date of mailing of the international search report

25 JAN 2001

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

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*Robert Schwartzman*  
ROBERT SCHWARTZMAN

Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/24129

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LADEROUTE et al. The inhibition of apoptosis by alpha-fetoprotein (AFP) and the role of AFP receptors in anti-cellular senescence. Anticancer Res. November-December 1994, Vol. 14, No. 6B, pages 2429-2438, especially pages 2433-2434.	1
Y	BENNETT et al. Similarity between natural and recombinant alpha-fetoprotein as inhibitors of estrogen-dependent breast cancer growth. Breast Cancer Res. Treat. September 1997, Vol. 45, No. 2, pages 169-179, see entire document.	1, 2
A	PALU et al. In pursuit of new developments for gene therapy of human diseases. J. Biotechnol. 1999, Vol. 68, pages 1-13, see entire document.	1-8

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/24129

### B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

STN: Medline, Biosis, Embase, CAPus

WEST: All databases

Search Terms: alpha-fetoprotein, AFP, apoptosis, programmed cell death